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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

EIJI SHIOJIRI, ET AL. : EXAMINER: KAM, C.M.

SERIAL NO: 09/926,391 :

FILED: JANUARY 8, 2002 : GROUP ART UNIT: 1653

FOR: MELANOCYTE-STIMULATING HORMONE INHIBITORS

DECLARATION UNDER 37 C.F.R.§1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VA 22313-1450

SIR:

(A) Now comes Eiji Shiojiri, who deposes and states that:

1. That I am a named inventor of the above-identified application.

2. That I am a graduate of Kyoto University, and received my master's degree in the field of chemistry, in the year 1989.

3. That I have been employed by Ajinomoto Co., Inc., for 17 years as a synthetic chemist in the field of specialty chemicals.

4. That I understand the English language or, at least, that the contents of the Declaration were made clear to me prior to executing the same.

5. That the following experiments were carried out by me or under my direct supervision and control.

(B) Now comes Yoshinobu Takino, who deposes and states that:

1. That I am a named inventor of the above-identified application.

2. That I am a graduate of Tohoku University, and received my master's degree in the field of pharmacy, in the year 1990.

3. That I have been employed by Ajinomoto Co., Inc., for 16 years as researcher in the field of cosmetic.

4. That I understand the English language or, at least, that the contents of the Declaration were made clear to me prior to executing the same.

5. That the following experiments were carried out by me or under my direct supervision and control.

(C) Experiments:

1. Tested compounds were synthesized as described below:

Abbreviations

HBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate

HOBt: 1-hydroxybezotriazole

TFA: trifluoroacetic acid

Fmoc: fluorenylmethoxycarbonyl

Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl

Mtr: 4-methoxy-2,3,6-trimethylphenylsulfonyl

Boc: t-butyloxycarbonyl

DMF: N,N-dimethylformamide

DCM: dichloromethane

Amino acid derivatives, synthetic reagents and resins used for synthesis

These were purchased from PerkinElmer Inc., Novabiochem (Merck Biosciences AG), AnaSpec Inc., Kokusan Chemicals Ltd., or Bachem AG.

Instruments

Peptide synthesizer - Protected peptide derivatives-resins were developed by Fmoc method using Applied Biosystems 433A peptide synthesizer (PerkinElmer) or peptide synthesizer ACT350 (AdvancedChemTech).

Synthesis of various peptide derivatives

Compound 1: D-2-Nal-Lys-Leu-NH2

Using peptide synthesizer ACT350 (AdvancedChemTech) regulated with Fmoc peptide synthesis protocol, 50 mg of resin carrier conjugated with Fmoc group 0.56mmol/g (Rink amide MBHA resin, Novabiochem) was applied to reaction chamber of the peptide synthesizer, following procedures were carried according to synthesis protocol of AdvancedChemTech.

(a) 500μl of 30% piperidine-DMF was added to carrier resin, mixture was stirred for 5 minutes and was excluded, and this procedure was repeated one more time.

(b) 1 ml of carrier resin was washed with DMF for one minute and was excluded, and this procedure was repeated 5 more times.

(c) Fmoc-Leu (112μmol), HBTU (112μmol), HOBt 1 hydrate (112μmol) and DIEA (224μmol) were stirred in DMF(1ml) for 3 minutes, obtained solution was added

by carrier resin, and resulted mixture was stirred for 60 minutes, and then solution was excluded.

(d) Carrier resin was washed by 600μl DMF for 1 minute, solution was excluded, and this procedure was repeated for 5 times. According to above described processes, Fmoc-Leu-NH was synthesized on carrier resin.

Next, after the processes of (a) and (b), condensation reaction was carried in the process (c) using Fmoc-Lys(Boc), then process (d) of washing carried, and Fmoc- Lys(Boc)-Leu-NH was synthesized on carrier.

Hereinafter, Fmoc-D-2-Nal was used in the process (c); similar processes were repeated: deviation from protection process of (a) and (b) were carried; then washing process was carried using DCM; resulted product was dried for 12 hours under decompressed; and then carrier resin which conjugates with side-chain protected peptide was obtained. Cleavage cocktail (Reagent K) 1ml was added and stirred for two hours; side-chain protection group was removed, and peptide was cut off from resin. As used herein Reagent K is a mixture consisting of TFA (82.5%), thio anisole (5%), water (5%), 1,2-ethane dithiol (2.5%), and thiophenol (5%).

After the separation of resin, obtained solution was added with ether about 10 ml; resulted white precipitates were separated with Kiriyama Rohto and washed with ether about 20ml. Next, total coarse peptide was dissolved with 0.05%TFA aqueous solution and purified with HPLC using reverse phase ODS column (GL science, Inertsil ODS-3, 25mmI.D. X 250mm). Peptide was eluted by linear gradient elution with 0.05% TFA aqueous solution gradually added by acetonitrile containing 0.05 % TFA and detected at 210nm; peptide was freeze-dried after separation and purification; and 13.2 mg of compound 1 was obtained.

Mass Spectrometry [MS]; m/z = 456.4 (M+H+)

Compound 2: D-2-Nal-Arg-Leu-OH

Fmoc-Leu -O-Resin binding 0.25mmol Fmoc-Leu was used as starting carrier resin. Using peptide synthesizer 433A (Applied Biosystems), Fmoc-Arg(Pbf)(1mmol) and Fmoc-D-2-Nal (1mmol) were used to elongate peptide chain from C-terminal according to the program controlled by Fmoc method; objective protected peptide resin was synthesized; protection group was cleaved; resulted compound was purified and freeze-dried; and 15.4 mg compound 2 as TFA salt form was obtained.

Compound 3: N-Ac-D-2-Nal-Arg-Leu-NH2

Synthetic procedure was performed with the same way of compound 1 except for that Fmoc-Arg(Pbf) was used instead of Fmoc-Lys(Boc) and the segment of acetic acid condensation was added after the synthetic program sequence; protection group was then cleaved; resulted compound was purified and freeze-dried; and 11.1 mg of compound 3 was obtained as TFA salt form.

Mass Spectrometry [MS]; m/z = 526.4(M+H+)

Compound 4: N-Butanoyl-D-2-Nal-Arg-Leu-NH2

Synthetic procedure was performed with the same way of compound 3 except for that butyric acid was used instead of acetic acid; protection group was then cleaved; resulted compound was purified and freeze-dried; and compound 4 was obtained as TFA salt form.

Compound 5: N-Lauroyl-D-2-Nal-Arg-Leu-NH2

Synthetic procedure was performed with the same way of compound 3 except for that lauric acid was used instead of acetic acid; protection group was then cleaved; resulted compound was purified and freeze-dried; and compound 5 was obtained as TFA salt form.

Compound 6: D-2-Nal-Arg-NH2

Synthetic procedure was performed with the same way of compound 1 except for that Fmoc-Leu was not used at the first condensation and Fmoc-Arg(Pbf) was used instead of Fmoc-Lys(Boc); protection group was then cleaved; resulted compound was purified and freeze-dried; and 23.7 mg of compound 6 was obtained as TFA salt form.

Mass Spectrometry [MS]; m/z = 371.3 (M+H+)

Compound 7: D-2-Nal-Arg-Trp-NH2

Synthetic procedure was performed with the same way of compound 2 except for that 455 mg of carrier resin binding 0.55mmol/g of Fmoc group (0.25mmol; Rink amide MBHA resin, Novabiochem) was used instead of Fmoc-Leu -O-Resin, and Fmoc-Trp (1 mmol), Fmoc-Arg(Pbf) (1 mmol) and Fmoc-D-2-Nal (1 mmol) were consequently used to elongate the peptide chain from C-terminal according to the Fmoc method program; and objective protected peptide-resin was synthesized; protection group was then cleaved; resulted compound was purified and freeze-dried; and compound 7 was obtained as TFA salt form.

Compound 8: D-Trp-Arg-Nle-NH2 (dWR-Nle-NH2)

Synthetic procedure was performed with the same way of compound 7 except for that

Fmoc-Nle was used instead of Fmoc-Trp and Fmoc-D-Trp was used instead of Fmoc-D-2-Nal; objective protected peptide resin was then synthesized; protection group was then cleaved; resulted compound was purified and freeze-dried; and compound 8 was obtained as TFA salt form.

Compound 9: D-Trp-N-Me-Arg-Leu-OH

Synthetic procedure was performed with the same way of compound 2 except for that Fmoc-N-Me-Arg(Mtr) was used instead of Fmoc-Arg(Pbf) and Fmoc-D-Trp was used instead of Fmoc-D-2-Nal; objective protected peptide resin was then synthesized; protection group was cleaved; resulted compound was purified and freeze-dried; and compound 9 was obtained as TFA salt form.

Compound 10: D-Trp-Arg-Leu-NH2(dWRL-NH2)

Synthetic procedure was performed with the same way of compound 7 except for that Fmoc-Leu was used instead of Fmoc-Trp and Fmoc-D-Trp was used instead of Fmoc-D-2-Nal; objective protected peptide resin was then synthesized; protection group was cleaved; resulted compound was purified and freeze-dried; and compound 10 was obtained as TFA salt form.

Compound 11: D-Trp-Arg-CHA-NH2

Synthetic procedure was performed with the same way of compound 10 except for that Fmoc-CHA (cyclohexyl alanine) was used instead of Fmoc-Leu; objective protected peptide resin was then synthesized; protection group was cleaved; resulted compound was purified and freeze-dried; and compound 11 was obtained as TFA salt form.

Compound 12: N-a-lauroyl-dWRL-NH2

Synthetic procedure was performed with the same way of compound 10 except for that the segment of lauric acid condensation was added after the synthetic program sequence; protection group was then cleaved; resulted compound was purified and freeze-dried; and compound 12 was obtained as TFA salt form.

Compound 13: D-Trp-N-Me-Arg-Leu-NH2

Synthetic procedure was performed with the same way of compound 10 except for that Fmoc-N-Me-Arg(Mtr) was used instead of Fmoc-Arg(Pbf); objective protected peptide resin was then synthesized; protection group was cleaved; resulted compound was purified and freeze-dried; and compound 13 was obtained as TFA salt form.

Compound 14: D-Trp-Arg-Leu-OH

Synthetic procedure was performed with the same way of compound 2 except for that Fmoc-D-Trp was used instead of Fmoc-D-2-Nal; objective protected peptide resin was then synthesized; protection group was cleaved; resulted compound was purified and freeze-dried; and compound 14 was obtained as TFA salt form.

Compound 15: D-Trp-Nle-NH2 (dW-Nle-NH2)

Synthetic procedure was performed with the same way of compound 8 except for that Fmoc-Arg(Pbf) was not used; objective protected peptide resin was then synthesized; protection group was cleaved; resulted compound was purified and freeze-dried; and compound 15 was obtained as TFA salt form.

Compound 16: D-Trp-Arg-OH(dWR-OH)

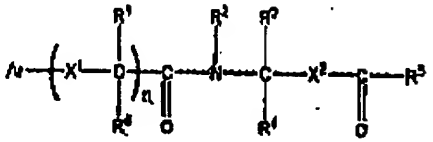
Fmoc-Arg(Mtr) -O-Resin binding 0.25mmol Fmoc-Arg(Mtr) was used as starting carrier resin. Using peptide synthesizer 433A (Applied Biosystems), Fmoc-D-Trp was condensed; objective protected peptide resin was synthesized; protection group was cleaved; resulted compound was purified and freeze-dried; and compound 16 as TFA salt form was obtained.

2. MSH antagonist assay:

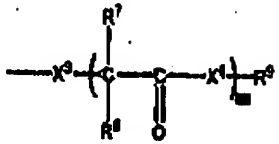
Synthesized compounds were examined as follows:

Melanophores (Melanin containing cells) prepared from the tail skin of the tadpole as described by [D1: Long-term Cultivation of Amphibian Melanophores: In vitro Ageing and Spontaneous Transformation to a Continuous Cell Line; Experimental Cell Research 149 (1983) 247-256] were separated and cultured, and those cells were used for alpha-MSH antagonist assay. Cells prepared as described by D1 were seeded onto 96-well plate (7500 cells/well), and each compound was examined for MSH-antagonizing activity using the plate as described by [D2: Design, synthesis, and biological activities of a potent and selective alpha-melanotropin antagonist; Int. J. Peptide Protein Res. 35, 1990, 228-234]. Activities were

determined as IC50 by checking color change of the cells using naked eye and microscope, and by determining the optical density at 630 nm wavelength. The results and the corresonding structures are shown below:



(1)



(2)

Category	Compound	Structure	Ar	X1	n	R1	R5	R2	R3	R4	X2	R5						IC50 by other screening method
												X3	R7	R8	X4	m	R9	
in current claim	ex.1	D-1-Nal-Arg-Leu-NH2	Naphthyl	single bond	1	Me	NH2	H	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	0.028pM
	ex.2	D-2-Nal-Arg-Leu-NH2	Naphthyl	single bond	1	Me	NH2	H	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	0.001pM
	ex.3	L-1-Nal-Arg-Leu-NH2	Naphthyl	single bond	1	Me	NH2	H	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	3.5pM
	ex.4	L-2-Nal-Arg-Leu-NH2	Naphthyl	single bond	1	Me	NH2	H	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	0.07pM
	1	D-2-Nal-Lys-Leu-NH2	Naphthyl	single bond	1	Me	NH2	H	H	Lys SC	single bond	NH	Leu SC	H	NH	1	H	0.2pM
	2	D-2-Nal-Arg-Leu-OH	Naphthyl	single bond	1	Me	NH2	H	H	Arg SC	single bond	NH	Leu SC	H	O	1	H	4.5pM
	3	N-Ac-D-2-Nal-Arg-Leu-NH2	Naphthyl	single bond	1	Me	NH-Ac	H	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	18pM
	4	N-Butanoyl-D-2-Nal-Arg-Leu-NH2	Naphthyl	single bond	1	Me	NH-butanoyl	H	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	Not tested
	5	N-Lauroyl-D-2-Nal-Arg-Leu-NH2	Naphthyl	single bond	1	Me	NH-lauroyl	H	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	Not tested
	6	D-2-Nal-Arg-NH2	Naphthyl	single bond	1	Me	NH2	H	H	Arg SC	single bond	NH	*	*	*	0	H	9pM
in original claim	7	D-2-Nal-Arg-Trp-NH2	Naphthyl	single bond	1	Me	NH2	H	H	Arg SC	single bond	NH	Trp SC	H	NH	1	H	Not tested
	8	D-Trp-Arg-Nle-NH2 (d NR-Nle-NH2)	Indolyl	methylene	1	H	NH2	H	H	Arg SC	single bond	NH	butyl	H	NH	1	H	0.07pM
	9	D-Trp-N-Me-Arg-Leu-OH	Indolyl	methylene	1	H	NH2	Me	H	Arg SC	single bond	NH	Leu SC	H	O	1	H	about 0.1pM
	10	D-Trp-Arg-Leu-NH2(dNRL-NH2)	Indolyl	methylene	1	H	NH2	H	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	0.2pM
	11	D-Trp-Arg-CHA-NH2	Indolyl	methylene	1	H	NH2	H	H	Arg SC	single bond	NH	CHA SC	H	NH	1	H	0.4pM
	12	N-a-lauroyl-dNRL-NH2	Indolyl	methylene	1	Me	NH-lauroyl	H	H	Arg	single	NH	Leu	H	NH	1	H	about 2pM

										SC	bond		SC						
13	D-Trp-N-Me-Arg-Leu-NH2	Indolyl	methylene	1	H	NH2	Me	H	Arg SC	single bond	NH	Leu SC	H	NH	1	H	3pM
14	D-Trp-Arg-Leu-OH	Indolyl	methylene	1	H	NH2	H	H	Arg SC	single bond	NH	Leu SC	H	O	1	H	No Activity (=>100pM)
15	D-Trp-Nle-NH2 (dW-Nle-NH2)	Indolyl	methylene	1	H	NH2	H	H	butyl	single bond	NH	*	*	*	0	H	No activity
16	D-Trp-Arg-OH(dWR-OH)	Indolyl	methylene	1	H	NH2	H	H	Arg SC	single bond	O	*	*	*	0	H	No activity

Indolyl=1H-Indol-3-yl
SC=Side chain
Nle=norleucine
CHA=cyclohexyl alanine

(D) I declare further that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

(E) Further Declarants saith not.

Eiji Shiojiri

Name: Eiji Shiojiri

19 June 2006

Date

Yoshinobu Takino

Name: Yoshinobu Takino

19 June 2006

Date